VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE SPECIFICATION:

Paragraph beginning on line 20 of page 14 has been deleted.

Paragraph beginning on line 13 of page 15 has been amended as follows:

Figures 7A and 7B ~~8A and 8B~~ show that decreased dietary protein intake correlates with less mitochondrial DNA damage in control and apoE mice. Control and apoE mice were fed either a 16% or 24% protein diet for 4 weeks, commencing at 6 weeks of age, and mitochondrial DNA damage was assesssed by QPCR. **Figure** 7A ~~8A~~ illustrates the relative amplification of control and apoE mitochondrial DNA in aortas relative to the 24% protein fed control group. Less amplification product signifies increased mitochondrial DNA damage. Asterisks (*) indicate a significant difference between the 24% and 16% protein fed mice. Students t' test P values are in parentheses. **Figure** 7B ~~8B~~ illustrates the relative amplification of control and apoE mitochondrial DNA in the left ventricle relative to the 24% protein fed control group. Less amplification product signifies increased mitochondrial DNA damage. Asterisks (*) indicate a significant difference between the 24% and 16% protein fed mice. Students t' test P values are in parentheses.

Paragraph beginning on line 8 of page 16 has been amended as follows:

Figure 8 ~~9~~ shows that lipid peroxidation is significantly increased in apoE compared to control mice, but lipid peroxidation is significantly increased in control mice fed the western (21% fat) diet. Bar

graph illustrates the level of lipid peroxidation in apoE and control mice fed chow (4% fat) or western (21%) diets. Values are expressed relative to the chow fed control mice. While apoE had significantly higher levels of lipid peroxidation compared to control mice regardless of diet, there were no significant differences in the level of lipid peroxidation among the apoE fed the chow or western diets. By contrast, the control mice fed the western diet had significantly higher levels of lipid peroxidation (indicated by "*") compared to control mice fed the chow diet.

Paragraph beginning on line 20 of page 16 has been amended as follows:

Figure 9 ~~10~~ shows QPCR of age-matched normal and atherosclerotic aortic mtDNA from humans. The 16.2 kb (upper row) and 0.22 kb (bottom row) products represent the long and short human QPCR amplicons, respectively. Methods for QPCR from human mtDNA have been previously described (Ballinger, 1996, 1999; Yakes, 1997).

Paragraph beginning on line 5 of page 17 has been amended as follows:

Figures 10A and 10B ~~11A and 11B~~ show that hybrid apoE (-/-), SOD2 (+/-) mice have increased atherosclerotic lesions and mtDNA damage. In **Figure 10A ~~11A~~**, whole aortas from hybrid apoE (-/-), SOD2 (+/-) and apoE (-/-) littermates were oil-red-O stained. The upper left panel shows an oil-red-O stained aorta from a 17 week-old apoE (-/-), SOD2 (+/-) male (4% fat diet), and the upper right panel shows the result from an apoE (-/-) male littermate (4% fat diet). Lower left and right panels show stained aortas from 34 week-old apoE (-/-), SOD2 (+/-) and apoE (-/-) littermates, respectively. Black arrows indicate presence of large atherosclerotic lesions in the apoE (-/-), SOD2 (+/-) and apOE (-/-), (not yet present in the 17-week-old apoE (-/-) littermate), and the red

arrow in each panel point to an example of smaller lesions formed at arterial branches, that were more frequenct and characteristic of the apoE (-/-), SOD2 (+/-) mice. **Figure** 10B ~~11B~~ is a bar graph depicting atherosclerotic lesion frequencies from apoE (-/-), SOD2 (+/-) and apoE (-/-) mice whole aortas, visualized by oil-red-O staining (n=4).

Paragraph beginning on line 1 of page 18 has been amended as follows:

Figure 11 ~~12~~ shows the increased incidence of mitochondrial DNA damage in patients with risk factors for myocardial infarction. Blood samples were taken at the time of cardiac catherization to measure mitochondrial DNA damage by QPCR. For this figure, values of mitochondrial DNA damage above the mean for a "normal" population were considered increased. The bar graphs show the percentage of patients in each category with increased mitochondrial DNA damage. The occurrence of increased mitochondrial DNA damage is more frequent in subjects with hypertension, cigarette smoking, diabetes and combinations of these risk factors for myocardial infarction.

Paragraph beginnng on line 12 of page 18 has been amended as follows:

Figure 12 ~~13~~ shows mitochondrial DNA damage before and after a 20 mile "training run". Blood samples were taken, a buffy coat was obtained and mitochondrial DNA damage is present immediately after the training run, this quickly returns to normal values as shown here.

Paragraph beginning on line 18 of page 18 has been amended as follows:

Figure 13 ~~14~~ shows that ultramarathoners had significantly less mitochondrial DNA damage after being fed a high fat meal than did the control subjects. In all cases, mitochondrial DNA damage was assessed

before, immediately after eating a high fat meal and 4-6 hours later. The observed mitochondrial DNA damage in controls reflects the immediate effects of dietary intake of ROS in the form of fats and cholesterol. The relative "protection" observed in the ultramarathoners may relate to an upregulation of anti-oxidant defense mechanisms in these individuals.

Paragraph beginning on line 6 of page 19 has been amended as follows:

Figure 14 ~~1-5~~ shows that ultramarathoners had significantly less protein damage after being fed a high fat meal than did the control subjects.

Paragraph beginning on line 3 of page 53 has been amended as follows:

Aortic tissues from the hypercholesteremic apoE mice fed the chow diet had significantly increased levels of mitochondrial DNA damage compared to controls ($P < 0.05$; data not shown ~~Figure and 7A~~). These differences were observed in both the 10 and 34 week old mice, which clearly showed that apoE aortas sustained greater mitochondrial DNA damage *in vivo* compared to "healthy" mice. The 10 week old apoE aorta yielded a 61% decrease in amplification (decreased amplification correlates with increased DNA damage) relative to the zero class control, or an estimated 0.582 ± 0.123 mitochondrial DNA lesions/10 kilobases (kb) compared to 0.0 ± 0.198 lesions/10 kb in the 10 week old control mouse ($P = 0.018$). Similarly, the 34 week old apoE group had a three-fold increase in estimated mitochondrial DNA damage compared to the age matched control group (1.325 ± 0.257 lesions/10 kb versus 0.453 ± 0.162 lesions/10 kb; $P = 0.007$). These same patterns were also observed in tissue of the left ventricle of the heart (data not shown) ~~heart tissue (left ventricle; Figure 7B)~~. For example, the 10 week old apoE mice had a 67%

decrease in amplification compared to the zero class control (0.685 ± 0.093 lesions/10 kb versus 0.0 ± 0.048 lesions/10 kb; $P < 0.001$), while the 34 week old apoE mice sustained approximately 4-fold higher estimated mitochondrial DNA lesions compared to the age-matched controls (0.819 ± 0.151 lesions/10 kb versus 0.213 ± 0.295 lesions/10 kb, $P = 0.056$).

Paragraph beginning on line 4 of page 54 has been amended as follows:

The effects of the western diet were also assessed in apoE and control mice ~~(Figure 7B)~~. In the control mice, the western diet was associated with higher levels of mitochondrial DNA damage in the 10 week-old control group aortas compared to chow fed control mice (0.31 ± 0.17 lesions/10 kb versus 0.0 ± 0.20 lesions/10 kb, western and chow, respectively), however, this difference was less significant ($P = 0.25$). Such differences were less obvious in the 34 week old control mice (i.e. 0.45 ± 0.16 lesions/10 kb and 0.49 ± 0.25 lesions/10 kb in chow versus western, respectively, $P = 0.90$). Similarly, the 10 week old control mice showed increased estimated mitochondrial DNA damage with the western diet in heart (~~Figure 7B;~~ 58% decrease in amplification. 0.554 ± 0.23 lesions/10 kb and 0.0 ± 0.05 lesions/10 kb in western versus chow, respectively), yet similar with the results seen in the aorta, no obvious differences were seen in the 34 week old control mice (0.21 ± 0.29 lesions/10 kb and 0.25 ± 0.21 lesions/10 kb in chow versus western, respectively). By contrast, no apparent increases in mitochondrial DNA damage accompanied the western diet in either aorta or heart tissues from apoE mice. Hence, increased dietary fat appeared to be associated with increased mitochondrial DNA damage in the 10 week old control mice only.

Paragraph beginning on line 20 of page 55 has been amended as follows:

Because no significant differences in mitochondrial DNA damage were observed between the chow and western diets with matched genotype and age, the effects of protein in the 10 week old mice were investigated. Both apoE and c57B1 control mice were fed either 16% or 24% protein diets for 4 weeks, commencing at 6 weeks of age. While both diets resulted in apoE still having greater levels of mitochondrial DNA damage compared to controls (aorta $P = 0.015$; heart $P = 0.005$), the lower protein diet resulted in a significant decrease in damage in both control ($P = 0.007$) and apoE ($P < 0.001$) in aorta (Figure 0.21 lesions/10 kb in chow versus western, respectively). By contrast, no apparent increases in mitochondrial DNA damage accompanied the western diet in either aorta or heart tissues from apoE mice. Hence, increased dietary fat appeared to be associated with increased mitochondrial DNA damage in the 10 week old control mice only.

Paragraph beginning on line 20 of page 55 has been amended as follows:

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associated with decreased mitochondrial DNA damage in both types of mice.

Paragraph beginning on line 8 of page 58 has been amended as follows:

Lipid peroxidation levels were measured by determining the levels of MDA and 4-HNE. The hypercholesteremic apoE mice had significantly increased levels of lipid peroxides compared to control mice ($P < 0.05$). However, in contrast with the results observed with the total cholesterol levels, the degree of lipid peroxidation did not significantly incerase in apoE mice on the western compared to the chow diet (Figure 8 ~~9~~). By contrast, the control mice fed the western diet sustained increased lipid peroxidation compared to the chow diet ($P < 0.05$). Hence, while the apoE mice always had the highest levels of lipid peroxides compared to the control mice, the degree of lipid peroxidation in apoE did not increase when fed the western diet compared to the chow diet.

Paragraph beginning on line 4 of page 60 has been amended as follows:

QPCR analysis revealed significantly increased mtDNA damage in atherosclerotic tissues compared with healthy control group aortas (Figures 9 ~~10~~ and Table 8). By contrast, a marker for nuclear DNA damage, the *β-globin* gene cluster, did not sustain significant damage in atherosclerotic aorta compared with controls ($P = 0.15$). Consequently, the mtDNA sustained significant and preferential damage in human atherosclerotic aortas. These findings were consistent with the notion that mtDNA damage is an integral feature of atherogenesis. Because increased mtDNA damage was a consistent feature in both human and mouse atherogenesis, it was hypothesized that mitochondrial dysfunction is an

important event in atherogenesis, possible modulating the phenotype of vascular cells that are integral to atherosclerotic lesion formation.

Paragraph beginning on line 11 of page 68 has been amended as follows:

For analysis of atherogenesis and mtDNA damage, apoE (-/-), SOD2 (+/-) mice and apoE (-/-) controls were fed a 4% fat diet and sacrificed at 17 weeks of age. Assessment of functional SOD2 activity in hybrid apoE (-/-), SOD2 (+/-) aortas demonstrated a 44% reduction of activity compared with "normal" apoE (-/-) mice (data not shown; $P < 0.001$). Histological examination of atherosclerotic lesion development in these mice revealed that apoE (-/-), SOD2 (+/-) mice developed earlier lesions at physiologically relevant sites, including in the ascending aorta at the origin of the cerebral vessels. However, the most impressive finding was that at 17 weeks of age, oil-red-O positive lesions were present throughout the descending aorta at virtually every branch point of the spinal arteries in the apoE (-/-), SOD2 (+/-) mice (**Figure 10A ~~11A~~**). Analogous lesions were rare in both 17-week-old and 34-week-old apoE (-/-) mice. Overall, the apoE (-/-), SOD2 (+/-) mice had 2.5 fold as many atherosclerotic lesions ($P - 0.02$) compared with apoE (-/-) littermates (**Figure 10B ~~11B~~**). Although the small size of atherosclerotic lesions at the branch points precluded direct measurement of mtDNA damage at these sites, quantification of aortic mtDNA damage through QPCR revealed increased aortic mtDNA damage in the apoE (-/-), SOD2 (+/-) mice compared with their age-matched apoE (-/-) littermates (**Table 8**; $P = 0.006$). Thus, while providing further evidence that mitochondrial dysfunction resulting from oxidative mtDNA damage is important in atherogenesis, these finding also support the notion that, under normal

circumstances, decreased expression of SOD2 in response to turbulent flow at arterial branch points promotes atherogenesis at these sites.

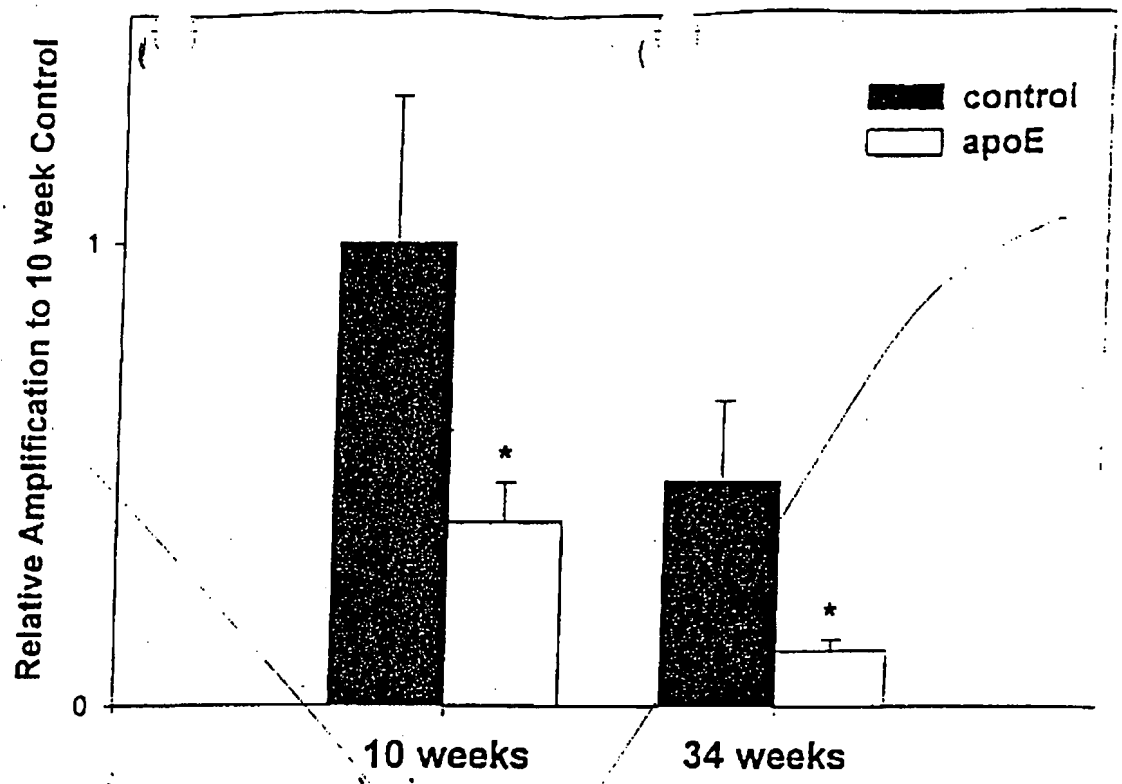
Paragraph beginning on line 9 of page 77 has been amended as follows:

Patient who were undergoing cardiac catherization were recruited for an *in vivo* study of mitochondrial DNA damage. All signed informed consent for an additional blood sample to be taken at the time of cardiac catherization. In all, there were 75 patients. White blood cells were isolated by standard techniques (the "buffy coat" preparation), and DNA (both nuclear and mitochondrial) was isolated. Mitochondrial DNA damage was determined by quantitative PCR using both "short mitochondrial DNA fragments" and damage within the β-globin gene as controls. Figure 11 ~~12~~ shows the increased incidence of mitochondrial DNA damage in patients grouped according to risk factors associated with myocardial infarction.

Paragraph beginning on line 18 of page 78 has been amended as follows:

To examine further the extent of mitochondrial DNA damage under a variety of *in vivo* conditions, 5 individuals who are "ultramarathoners" and an age-matched group of 6 individuals who are healthy and active, but not ultramarathoners, were examined. First, mitochondrial DNA damage was measured before and after a 20 mile "training run" by the ultramarathoners. Blood samples were taken, a buffy coat was obtained, and mitochondrial DNA damage was assessed. Figure 12 ~~13~~ shows that there is a mild increase in mitochondrial DNA damage just after the run, which returns to normal within 4 hours. On a different day, the ultramarathoners and the control subjects were fed a high fat meal. The high fat meal comprised the "El Grande Platter" at a

local Mexican food restaurant, which contained approximately 3000 calories, of which approximately 60-70% were fat calories. Blood samples were taken just before the meal, and 2 and 6 hours after. Buffy coats and DNA isolation were performed. Mitochondrial protein damage was also quantitated using white blood cell mitochondrial protein as a source. Figures 13 and 14 ~~14 and 15~~ show that there is a gradual, small increase in mitochondrial DNA damage in the controls, and increased mitochondrial protein damage at 2 hours that decreases at 4 hours. In the ultramarathoners, mitochondrial DNA damage and protein damage actually decreased to below baseline levels at 2 hours and begins returning to baseline by 6 hours. Presumably, since the ultramarathoners are subjected to increased oxidative stress when they run, their anti-oxidant protective systems are more effective. Thus, when exposed to a stimulus for DNA damage, the ultramarathoners have even less damage than normal. Subsequently, their antioxidant systems are activated and decrease their oxidant levels to below resting levels.



DELETED

FIG. 7A

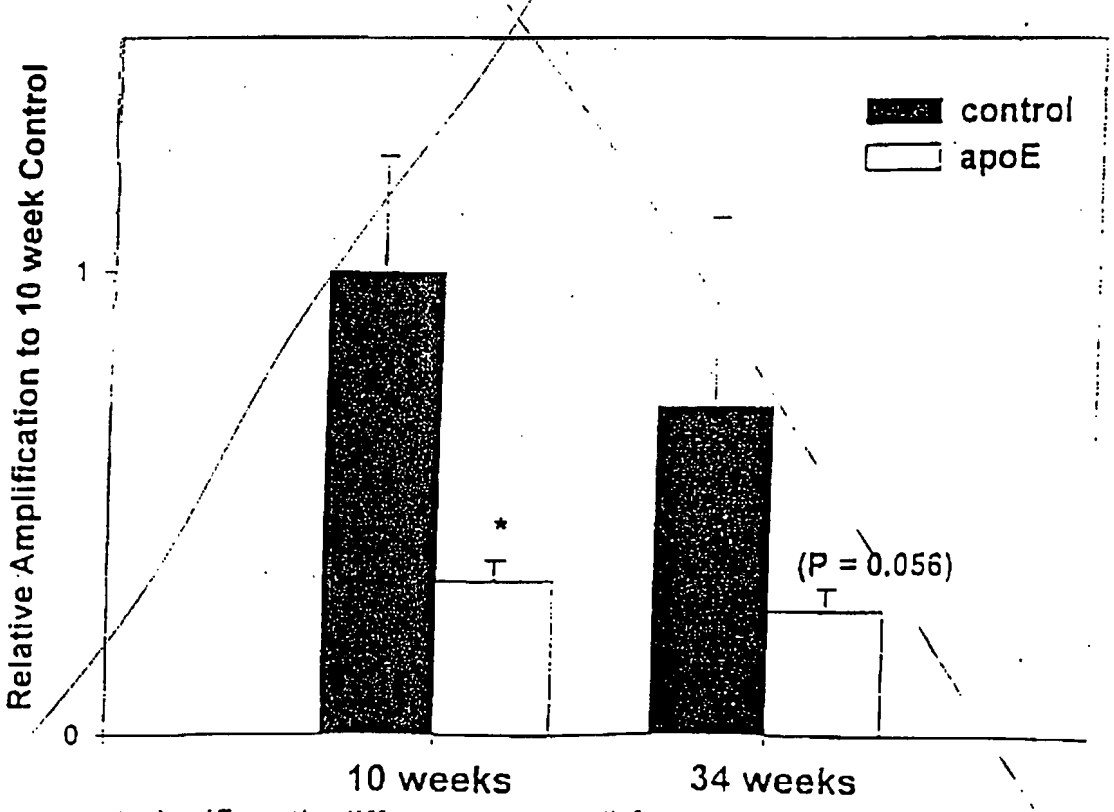


FIG. 7B

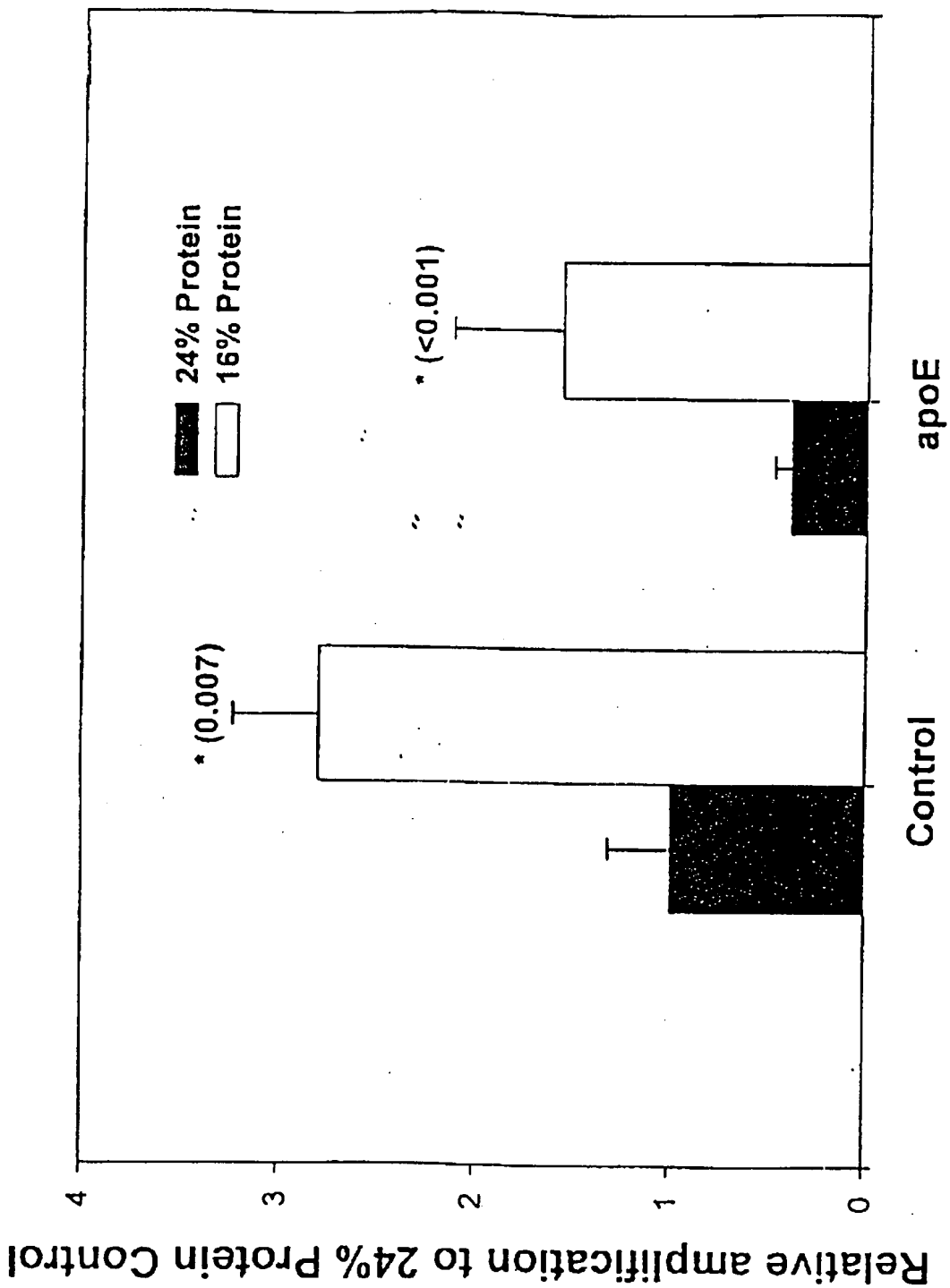

Relative amplification to 24% Protein Control
0
1
2
3
4
24% Protein
16% Protein
* (0.007)
* (<0.001)
Control
apoE

FIG. 7A ~~8A~~

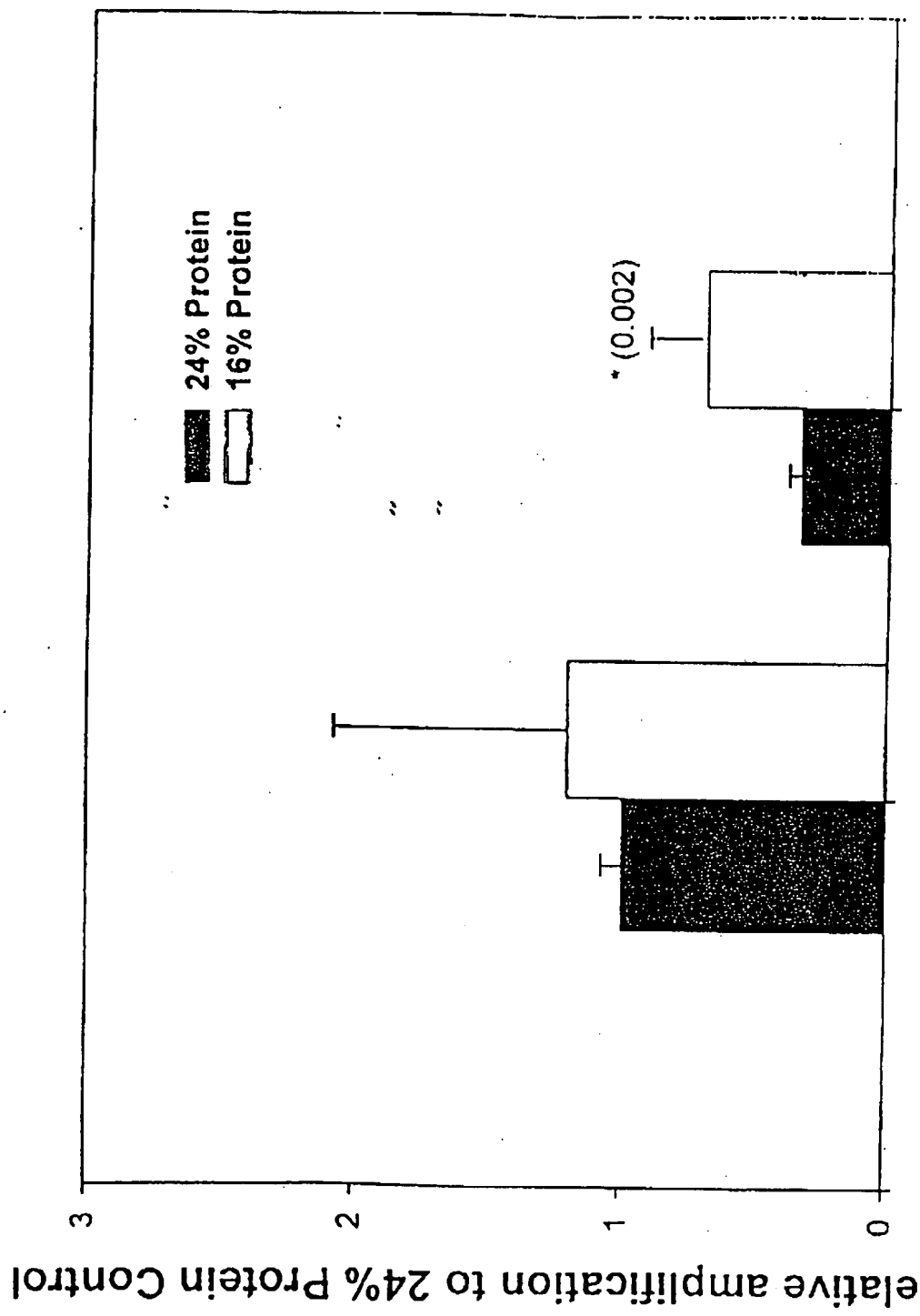
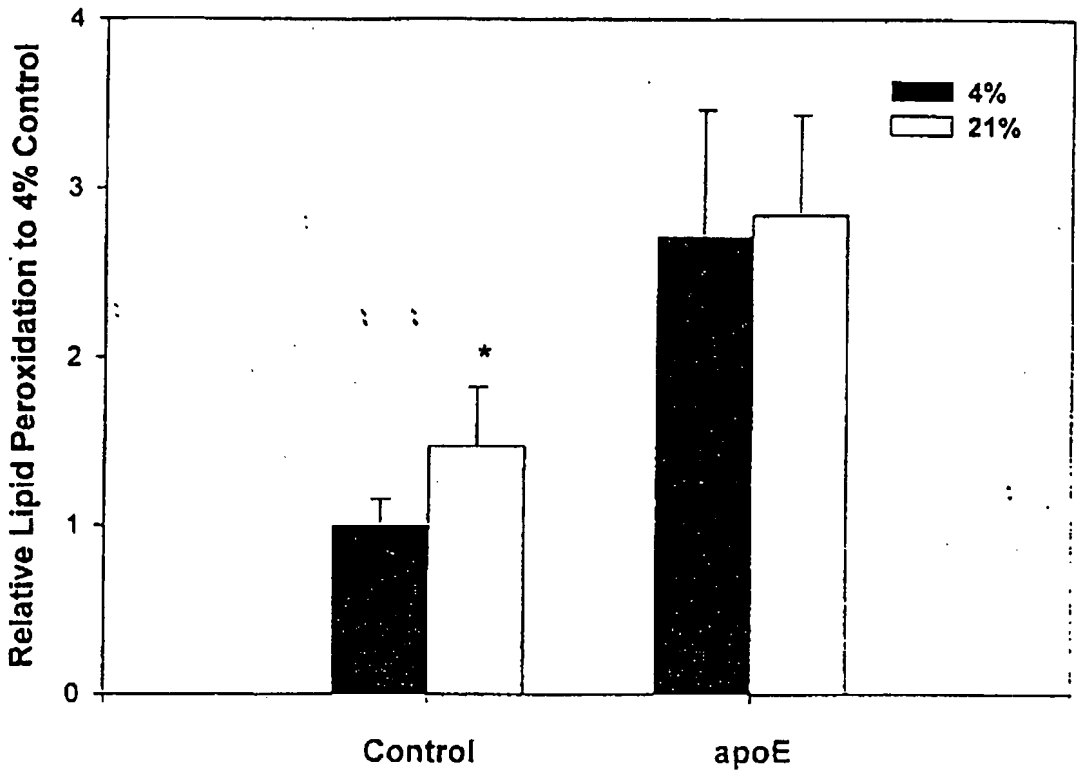

Relative amplification to 24% Protein Control
0
1
2
3
Control
apoE
* (0.002)
24% Protein
16% Protein

FIG. 8B



* significantly different (P < 0.05) from 4% diet

FIG. 9

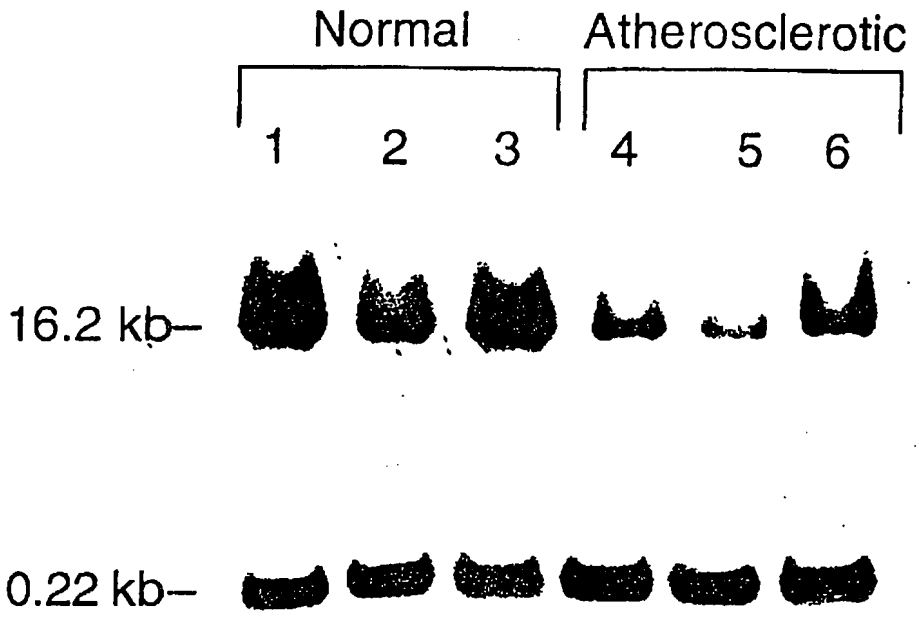
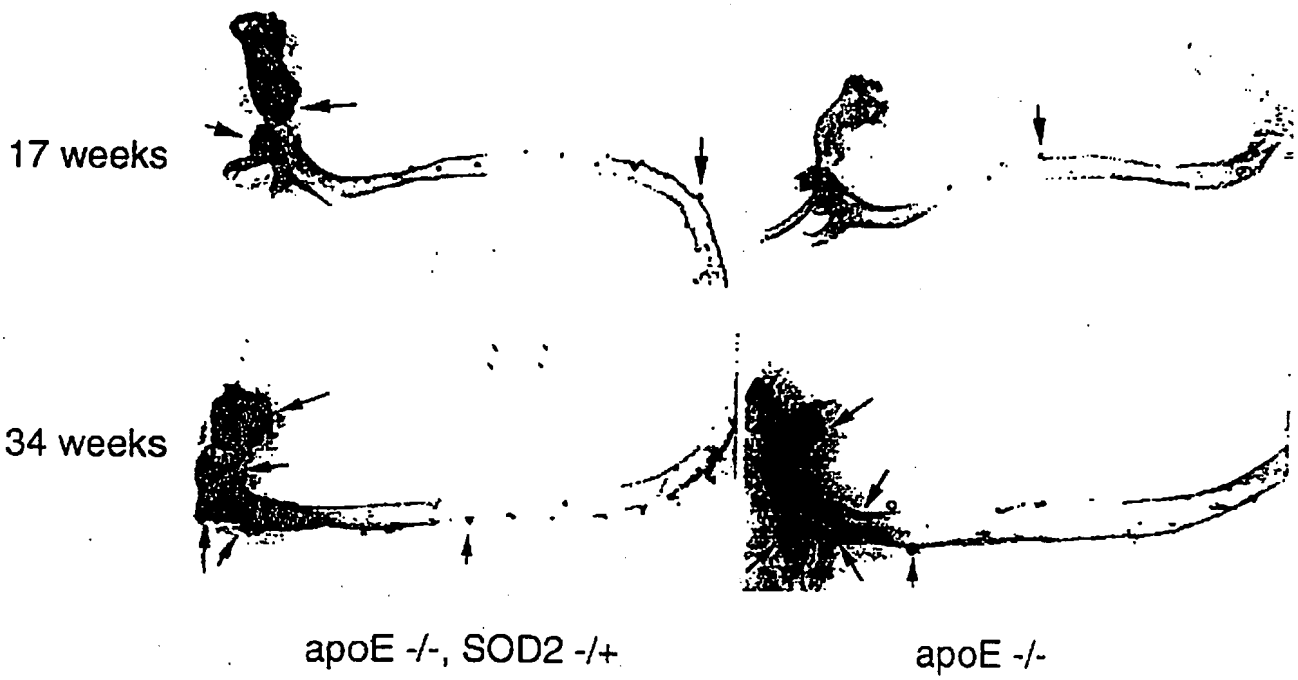


Figure 10

A


17 weeks
34 weeks
apoE -/-, SOD2 -/+
apoE -/-

B

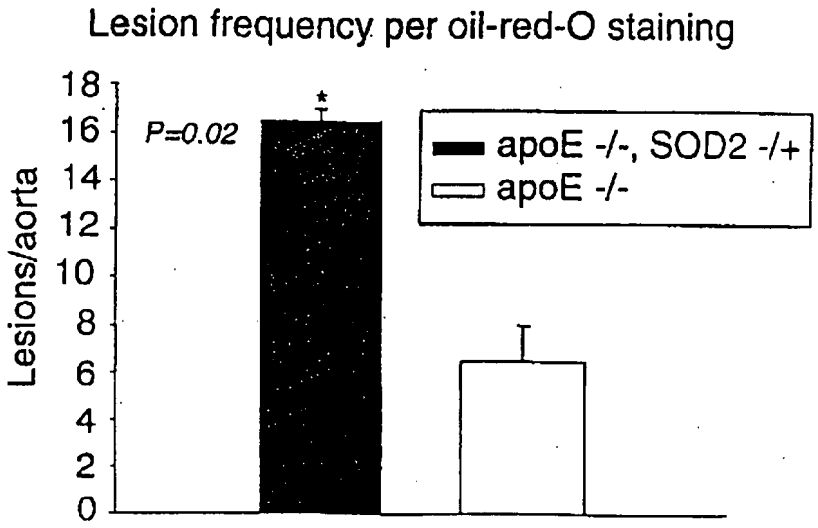

Lesion frequency per oil-red-O staining
Lesions/aorta
18
16
14
12
10
8
6
4
2
0
P=0.02
*
apoE -/-, SOD2 -/+
apoE -/-

Figure 11

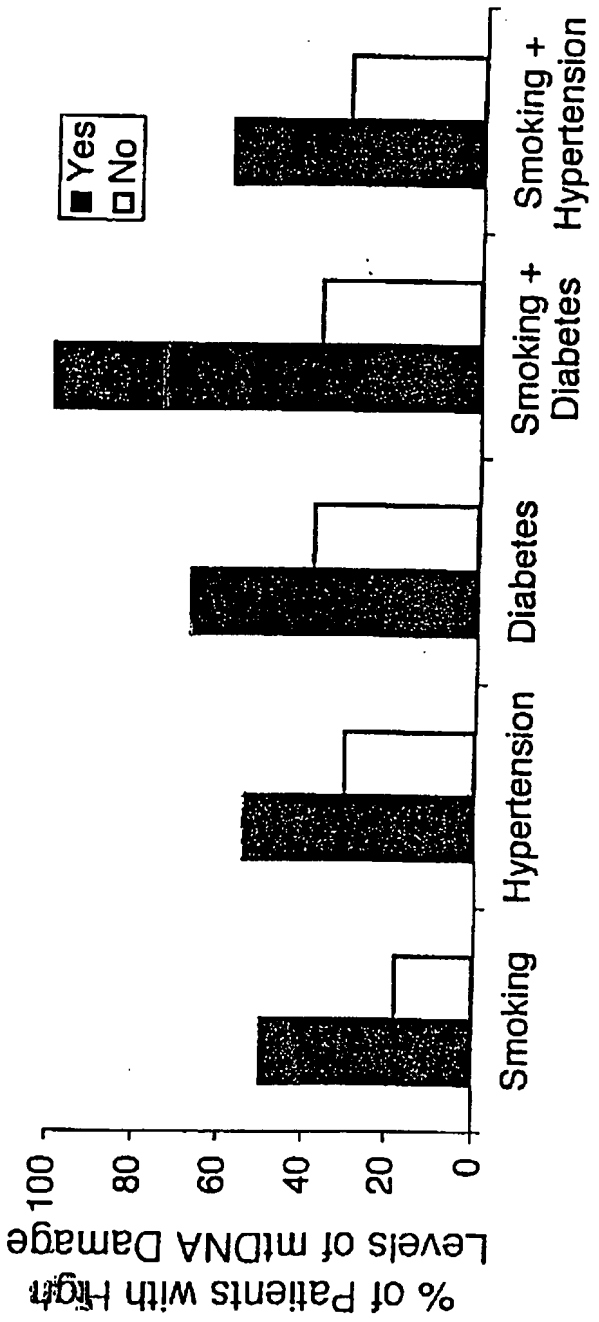

% of Patients with High Levels of mtDNA Damage
0
20
40
60
80
100
Smoking
Hypertension
Diabetes
Smoking + Diabetes
Smoking + Hypertension
Yes
No

Fig 1K

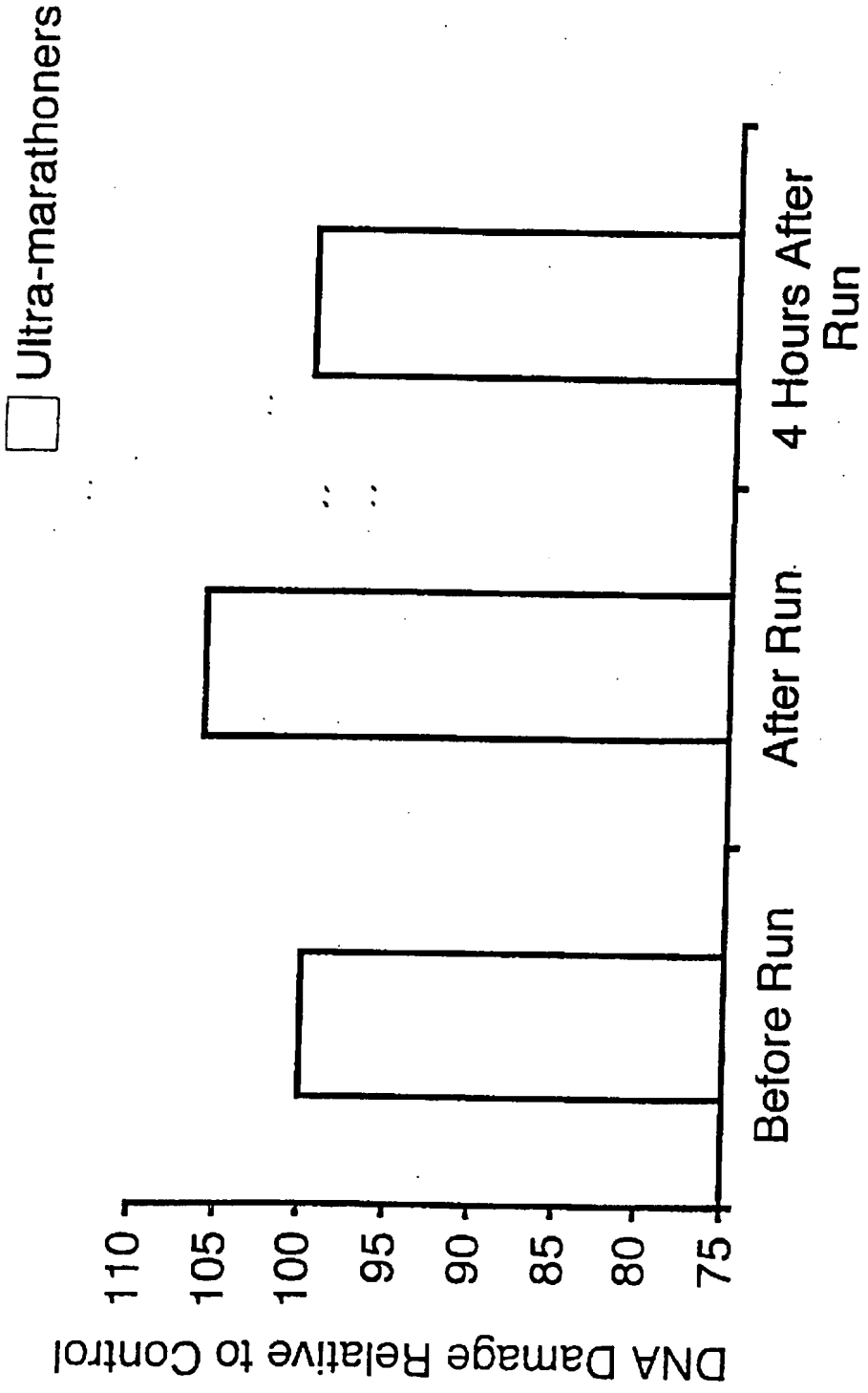

Ultra-marathoners
DNA Damage Relative to Control
110
105
100
95
90
85
80
75
Before Run
After Run
4 Hours After Run

Fig 12/13

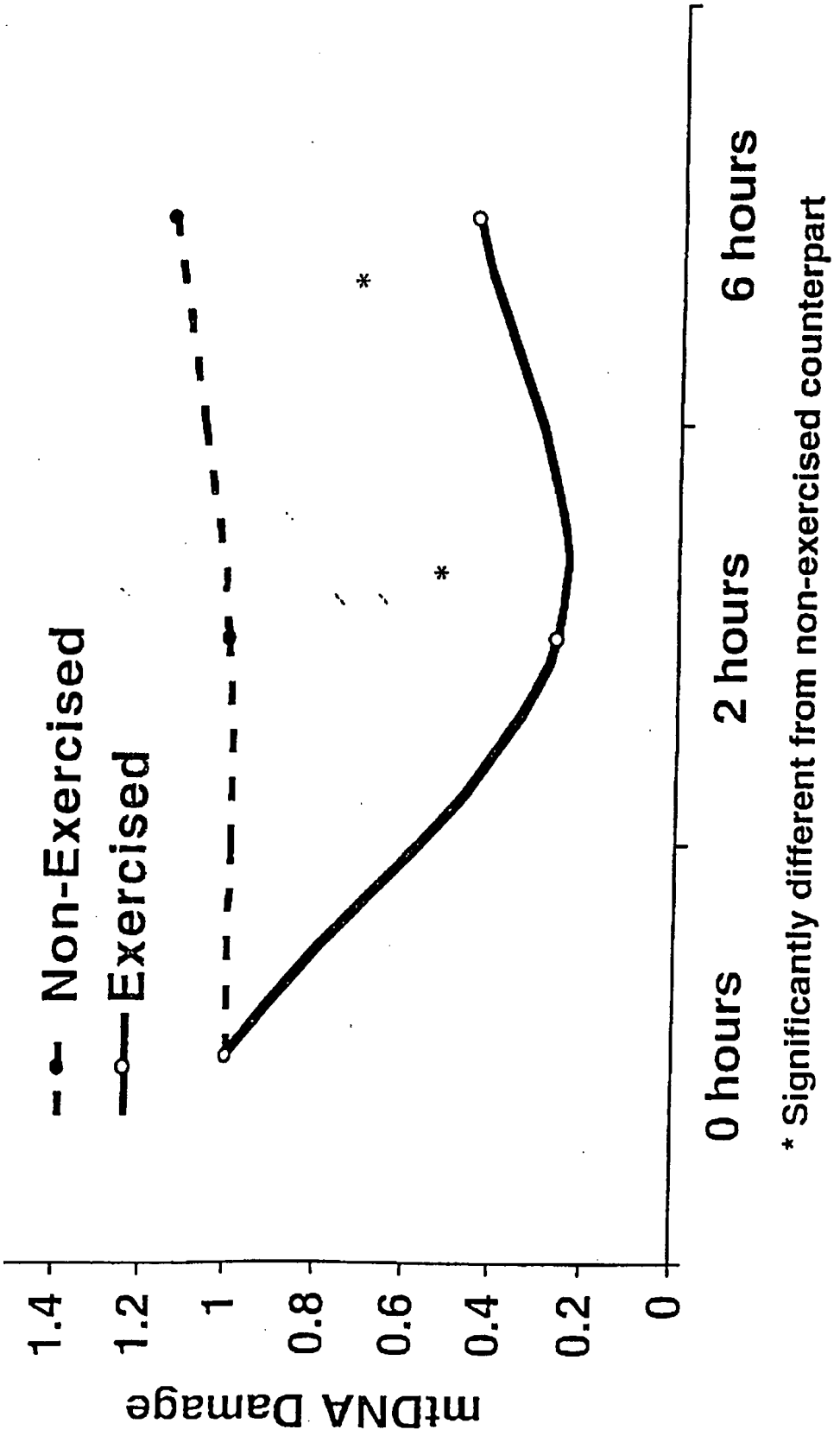

mtDNA Damage
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 hours
2 hours
6 hours
Non-Exercised
Exercised
* Significantly different from non-exercised counterpart

Fig 14

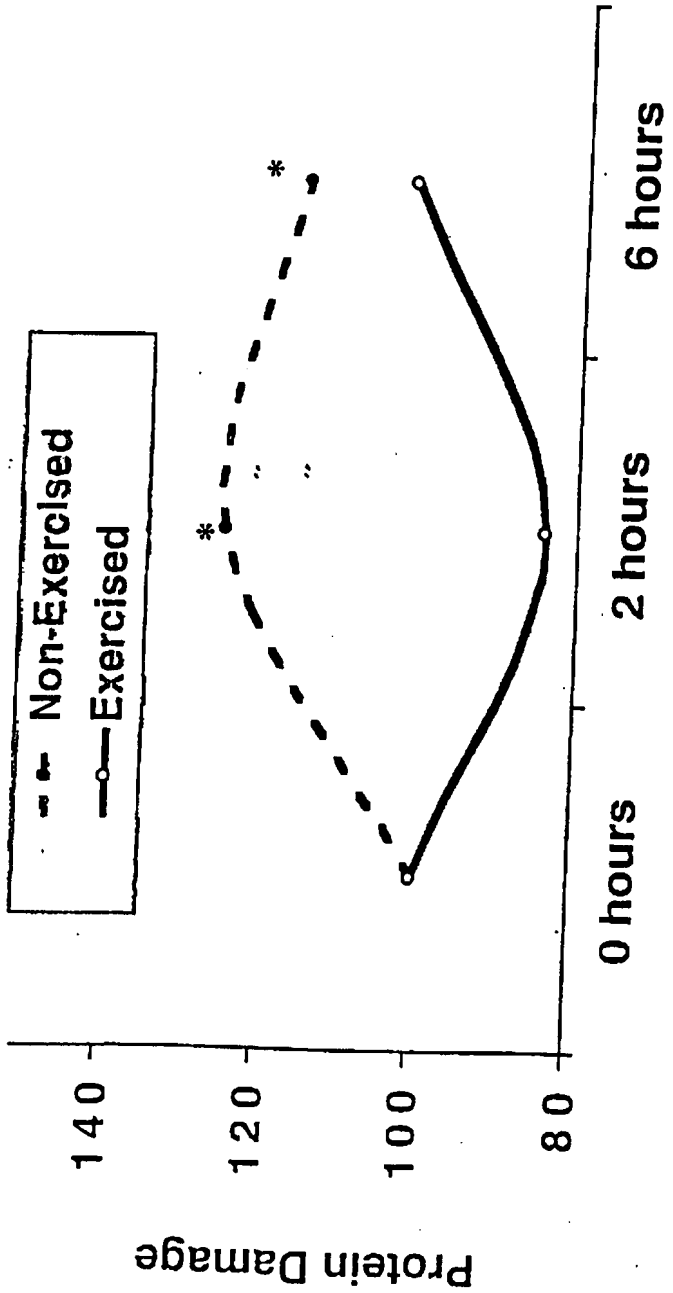

Non-Exercised
Exercised
Protein Damage
140
120
100
80
0 hours
2 hours
6 hours
* Significantly different from non-exercised counterpart

Figure 14/15